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The Temporal Control of Wee1 mRNA Translation during *Xenopus* Oocyte Maturation Is Regulated by Cytoplasmic Polyadenylation Elements within the 3′-Untranslated Region

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The Wee1 protein tyrosine kinase is a key regulator of cell cycle progression. Wee1 activity is necessary for the control of the first embryonic cell cycle following the fertilization of meiotically mature *Xenopus* oocytes. Wee1 mRNA is present in immature oocytes, but Wee1 protein does not accumulate in immature oocytes or during the early stages of progesterone-stimulated maturation. This delay in Wee1 translation is critical since premature Wee1 protein accumulation has been shown to inhibit oocyte maturation. In this study we provide evidence that Wee1 protein accumulation is regulated at the level of mRNA translation. This translational control is directed by sequences within the Wee1 mRNA 3′-untranslated region (3′ UTR). Specifically, cytoplasmic polyadenylation element (CPE) sequences within the Wee1 3′ UTR are necessary for full translational repression in immature oocytes. Our data further indicate that while CPE-independent mechanisms may regulate the levels of Wee1 protein accumulation during progesterone-stimulated oocyte maturation, the timing of Wee1 mRNA translational induction is directed through a CPE-dependent mechanism.

Key Words: **Wee1; mRNA; cytoplasmic polyadenylation; translation; *Xenopus;* oocyte; cell cycle; gene expression.**

INTRODUCTION

Embryonic development requires the strict temporal and spatial coordination of cell division. A key regulator of cell cycle progression is the maturation-, or M-phase-, promoting factor (MPF). MPF is composed of the serine/threonine kinase cdc2 and an associated cyclin B protein (Draetta *et al.*, 1989; Dunphy *et al.*, 1988; Gautier *et al.*, 1988, 1990). MPF activity is regulated both by the availability of cyclin B protein and by phosphorylation of cdc2 (Morgan, 1997; Pines, 1995). The Wee1 tyrosine kinase has been shown to inhibit MPF activity by phosphorylating cdc2 on tyrosine 15 (McGowan and Russell, 1993; Mueller *et al.*, 1995; Parker and Piwnica-Worms, 1992). In fertilized *Xenopus* eggs, Wee1 has been proposed to be responsible for the extended duration of the first mitotic cell cycle (Murakami *et al.*, 1999; Walter *et al.*, 2000), which may be necessary for gamete fusion, initiation of the mitotic cell cycle, and establishment of the bilateral symmetry in the embryo (Kirschner and Gerhart, 1981).

Although Wee1 protein is present in eggs prior to fertilization, it is notably absent in immature oocytes. Wee1 protein does not accumulate in progesterone-stimulated oocytes until after the completion of meiosis I (Murakami and Vande Woude, 1998; Nakajo *et al.*, 2000). Previous studies have demonstrated that expressing Wee1 in immature oocytes prevented progesterone-stimulated germinal vesicle breakdown (GVBD) (Howard *et al.*, 1999; Murakami and Vande Woude, 1998; Nakajo *et al.*, 2000). Recently it was suggested that the absence of Wee1 in immature oocytes is necessary for meiosis since ectopic expression of Wee1 converted the meiotic cell cycle into a mitotic-like cell cycle (Nakajo *et al.*, 2000). Thus the correct timing of Wee1 protein expression during oocyte maturation is critical. The molecular basis for this carefully orchestrated pattern of Wee1 protein accumulation has not been determined.

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The cytoplasmic polyadenylation of specific maternally derived mRNAs has been shown to be an evolutionarily conserved mechanism for the regulation of mRNA translation (Gebauer and Richter, 1996; Richter, 1999; Verrotti *et al.*, 1996; Walker *et al.*, 1999). In immature *Xenopus* oocytes the mRNAs encoding several key regulators of cell cycle progression have short poly(A) tails and are translationally silent. Upon progesterone stimulation and reinitiation of meiosis, these mRNAs are polyadenylated and translated (McGrew *et al.*, 1989; McGrew and Richter, 1990; Paris and Richter, 1990; Sheets *et al.*, 1994; Stebbins-Boaz and Richter, 1994). The translational silencing or masking of maternal mRNAs does not appear to be simply due to the short poly(A) tail but rather appears to require active repression (de Moor and Richter, 1999; Robbie *et al.*, 1995; Standart *et al.*, 1990; Stutz *et al.*, 1998). It is not clear if the derepression and polyadenylation of masked mRNAs are distinct separate events or if they are interrelated in a complex multistep process (Standart *et al.*, 1990; Stutz *et al.*, 1998).

In the 3′-untranslated region (UTR) of many translationally regulated *Xenopus* mRNAs, a *cis* element has been identified in close proximity to the ubiquitous polyadenylation hexanucleotide (AAUAAA) which is necessary for progesterone-dependent polyadenylation. This *cis* element has been termed the cytoplasmic polyadenylation element (CPE) that has a general sequence of $U_{4\text{-}5}A_{1\text{-}2}U_{1\text{-}2}$ (Fox *et al.*, 1989; McGrew *et al.*, 1989; Paris and Richter, 1990; Stebbins-Boaz and Richter, 1994). A CPE-binding protein (CPEB) has been shown to interact specifically with CPE sequences (Hake *et al.*, 1998; Hake and Richter, 1994; Stebbins-Boaz *et al.*, 1996). In *Xenopus*, as well as in clams and mice, CPE sequences and the *trans*-acting CPEB protein have been implicated in both translational repression and translational stimulation of specific mRNAs (Barkoff *et al.*, 2000; de Moor and Richter, 1999; Minshall *et al.*, 1999; Stebbins-Boaz *et al.*, 1999; Stutz *et al.*, 1998; Tay *et al.*, 2000; Walker *et al.*, 1999). Several maternal mRNAs have been identified which have one (e.g., Mos, cyclin A1, G10, B4) or more (e.g., cyclin B1, cyclin B2, cdk2) CPEs within the terminal 70 nucleotides of their respective 3′ UTRs. However, the extent and timing of both cytoplasmic polyadenylation and translation of CPE-containing mRNAs are variable (Ballantyne *et al.*, 1997; de Moor and Richter, 1997; Sheets *et al.*, 1994) suggesting that additional mechanisms may cooperate to orchestrate the strict temporal pattern of maternal mRNA translation.

In this study we have characterized the role of *cis* sequences within the Wee1 mRNA 3′ UTR in the temporal regulation of Wee1 mRNA translation during *Xenopus* oocyte maturation. We demonstrate that in response to progesterone, the Wee1 mRNA is polyadenylated coincident with the accumulation of Wee1 protein, consistent with a role for cytoplasmic polyadenylation in the regulation of Wee1 mRNA translation. We identify three CPE sequences within the Wee1 mRNA 3′ UTR and demonstrate that these CPE sequences contribute to translational repression in immature oocytes and are essential for the temporal control of progesterone-stimulated Wee1 mRNA translation in maturing oocytes.

MATERIALS AND METHODS

Plasmid Constructions and RNA Synthesis

Wee UTR constructs. PCR was used to isolate the complete 297 nucleotides of the 3′ UTR from the pAX-SV40 *Xenopus* Wee1 (pXe-Wee1) vector (P. R. Mueller, The University of Chicago, Chicago, IL) (Mueller *et al.*, 1995). Primers were designed to include a 5′ *Xba*I site [5′(+), GCGTC TAGAC CCCAT CAAAC CAAAT CCGTT] and a 3′ *Hin*dIII site [3′(−), GGCGC GAAGC TTACA CATTA AAATT TTTAT TTAAA A]. The resulting PCR product was blunt ligated into the *Sma*I site of pGEM4Z (Promega). Standard PCR mutagenesis was used to generate the series of Wee1 UTR mutant probes listed in Table 1. DNA sequencing was used to verify the integrity of the electrophoretic mobility shift assays (EMSA) sequences.

EMSA probes. A 5′ primer with an *Eco*RI site [5′(+), GCGGA ATTCG GGGCC TGGAC AAAAA C] and the M13(−) primer were used to amplify the last 79 nt of the Wee1 3′ UTR from the wild-type and mutant Wee UTR templates in the pGEM4Z vector. The PCR fragment was cut *Eco*RI/*Bam*HI and inserted into *Eco*RI/*Bam*HI-digested pGEM4Z. The total Wee1 EMSA probe length with polylinker was 92 nt. The mos EMSA probe was made by PCR amplifying the last 48 nt of the *Xenopus* Mos 3′ UTR. The Mos UTR(mut) had a mutation introduced by standard PCR mutagenesis that changed the CPE from UUUUAU to UUUggU.

Glutathione S-Transferase (GST) Wee UTR constructs. The GST open reading frame from pXen1 (MacNicol *et al.*, 1997) was isolated from accompanying 5′ and 3′ UTR sequences by subcloning into pGEM4Z to create pGEMGST. Briefly, the *Nco*I(blunt)/*Xba*I fragment from pXen1 was inserted into *Asp*718 (blunt)/*Xba*I-digested pGEM4Z. The Wee UTRs were excised from their respective plasmids described above and subcloned into pGEMGST.

GST β-globin UTR. Standard PCR techniques were used to insert an *Eco*RI site in place of the poly(A) tail of the 3′ UTR of β-globin from pXen1. The resulting 154-nucleotide β-globin 3′ UTR was excised with *Xba*I and *Eco*RI (blunt) and inserted into *Xba*I/*Hin*cII-digested pGEMGST.

GST–CPEB. *Xenopus* CPEB was RT-PCR amplified from total RNA from immature oocytes. Primers encoded a 5′ *Kpn*I site and a 3′ *Sma*I site. A 1708-bp coding sequence was obtained with an ATG start site and TAA stop site. This fragment was ligated into pXen2 (MacNicol *et al.*, 1997) to create an in-frame fusion construct, GST–CPEB. The truncated GST–$CPEB_{1\text{-}269}$ was constructed using primers to amplify the N-terminal 269 amino acids of CPEB and this was ligated into pXen2.

GST–BRaf was made by blunt ligating the *Hin*dIII/*Nde*I fragment of pSL-BRaf (a kind gift from Walter Kolch, Beatson Institute for Cancer Research, UK) into the *Sma*I site of pXen2.

In vitro transcription. Plasmids were linearized at appropriate restriction sites in the plasmid polylinker that were as close to the 3′ end of the Wee1 UTR as possible. For the full-length Wee1 UTR and most EMSA constructs this site was *Hin*dIII which added an additional “A” to the 3′ end. The GST constructs were linearized with *Pst*I which added “GACCUGCA” to the 3′ end of the transcribed RNAs. The truncated EMSA probes in Table 1 were

TABLE 1
Sequences of the Mutations That Were Used for This Study

Name	Sequence
WT	uuuauugacuuuuuuuuuuuuuuauuaucuuauuguc*uuuuaaauaaa*aauuuuaaugugua
EMSA 2&3	uuuauugacuuGCGGCCGCuuauuaucuuauuguc*uuuuaaauaaa*aauuuuaaugugua
EMSA 2 only	uuuauugacuuGCGGCCGCuuauuaucuuauuguc*uuuuaaauaaa*aauuGGaaugugua
EMSA 3 only	uuuauugacuuGCGGCCGCuuauuaucuuauuguc*uGGuaaauaaa*aauuuuaaugugua
EMSA no CPEs	uuuauugacuuGCGGCCGCuuauuaucuuauuguc*uGGuaaauaaa*aauuGGaaugugua
EMSA e&1	uuuauugacuuuuuuuuuuuuuauuaucuuauuguc*uuuu*
EMSA e only	uuuauugacuuuuuuuuuuuuuGGuaucuuauuguc*uuuu*
WeeUTR(1&2&3)	uuuauugacuuuGuuGuuuuuuauuaucuuauuguc*uuuuaaauaaa*aauuuuaaugugua
WeeUTR(e)	uuuauugacuuuuuuuuuuuuuGGuaucuuauuguc*uGGuaaauaaa*aauuGGaaugugua
WeeUTR(ϕ)	uuuauugacuuuGuuGuuuuuGGuaucuuauuguc*uGGuaaauaaa*aauuGGaaugugua
WeeUTR(hex$^-$)	uuuauugacuuuuuuuuuuuuuuauuaucuuauuguc*uuuuaaaGaaa*aauuuuaaugugua

Note. The last 60 nucleotides of the Wee1 3′ UTR before the poly(A) addition site are shown. Lowercase denotes wild-type sequence with the mutations shown in uppercase. The position of the maturation-type CPEs in the wild-type Wee1 UTR is underlined. The nuclear polyadenylation signal is shown in italics. The "EMSA e" and "EMSA e&1" are truncated due to linearization at the natural *Dra*I restriction site.

linearized at a naturally occurring *Dra*I site. All constructs were transcribed with SP6 RNA polymerase (Promega), as previously described (Melton *et al.*, 1984). The EMSA probes were synthesized without a 5′ diGTP cap.

Oocyte Culture and Injections

Oocytes were defolliculated by collagenase digestion (Sigma type II, 1 mg/ml) and placed in 1× modified Barth's saline solution plus Hepes (MBSH) with bovine serum albumin (1 mg/ml), Ficoll 400 (1 mg/ml), and antibiotics (Muslin *et al.*, 1993). Dumont stage VI immature oocytes were isolated and injected with 0.5–1 ng of RNA (Wee UTRs). To reveal progesterone-inducible translation, RNA-encoding GST–Wee UTR was titrated to 0.05–0.1 ng of RNA per oocyte in order to reduce the background level of translation, essentially the same as low-dose repression described in Barkoff *et al.* (2000). As a control to make sure that changes in translation were due to the inherent properties in the RNA, all RNA concentrations were equalized before injection and verified by Northern blot that they were all equally stable in immature and progesterone stimulated oocytes. Oocytes were stimulated where indicated with 2 μg/ml progesterone (Sigma) and the rate of GVBD was monitored morphologically by the appearance of a white spot on the animal hemisphere. For all experiments, except where indicated, pools of oocytes were harvested in proportion to the percentage of GVBD in each sample and immature control samples were prepared at the same time as the progesterone-stimulated oocyte samples. Results shown are representative experiments that were typically repeated three times with similar results.

Western Blot Analyses

Oocytes were lysed in 10 μl cold Nonidet P-40 buffer per oocyte (MacNicol *et al.*, 1993) and insoluble material and lipid were separated by centrifugation at 13,000*g* for 10 min at 4°C. Lysates were separated on 10 or 14% SDS–polyacrylamide gels (Novex) and transferred to a 0.2-μm nitrocellulose filter (Protran, Midwest Scientific). The filter was blocked with 5% nonfat dried milk in TBST [10 mM Tris (pH 7.5), 150 mM NaCl, 0.1% (v/v) Tween 20]. Filters were incubated with antibody and visualized with an appropriate HRP-linked secondary antibody using enhanced chemiluminescence (Amersham). Rabbit polyclonal antibody against GST (Z-5) was obtained from Santa Cruz Biotechnology, Inc. The antibody against the full-length Wee1 (P. R. Mueller, The University of Chicago) has been previously described (Mueller *et al.*, 1995). For analysis of both protein and RNA from the same oocyte samples, pools of oocytes were rapidly lysed in Nonidet P-40 buffer as above and then a portion was removed and immediately mixed with RNA STAT-60 (see below).

Polyadenylation Assay Using Northern Blot Analyses

Following the indicated treatments, total RNA was extracted from pools of 6–10 oocytes using 800 μl of RNA-STAT 60, according to the manufacturer's directions (Tel-Test B, Friendswood, TX) with an additional phenol/chloroform extraction. To obtain high-quality RNA, samples were reprecipitated with 8 M LiCl (as described in Sambrook *et al.*, 1989). Samples were resuspended in 22 μl loading buffer [1× Mops (0.04 M morpholinopropanesulfonic acid, pH 7.0, 12.5 mM sodium acetate, 1 mM EDTA), 18% formaldehyde, 45% formamide, 36 μg/ml ethidium bromide) and were resolved on a 1.5 or 2.2% (3:1 Nusieve:agarose) formaldehyde Northern gel. Samples were transferred to a 0.2-μm Nytran membrane (Schleicher and Schuell) and hybridized to a Wee1 3′-UTR-specific probe or GST-specific probe and visualized using the AlkPhos Direct system with CDP-*Star* chemiluminescence (Amersham Life Science). To confirm that any increase in mRNA size was specifically due to polyadenylation, RNA samples were treated with RNase H and oligo(dT) to eliminate any poly(A) tail prior to gel analysis. RNase H reactions were performed as previously described (Mercer and Wake, 1985). Briefly, 5–10 μg total RNA was incubated with 1 μl RNase H (1 U/μl, Boehringer Mannheim) in 40 μl digestion buffer (25 mM KCl, 0.5 mM EDTA, 10

mM Tris, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol) and the samples were incubated for 30 min at 37°C in the presence or absence of 0.3 μg of oligo(dT_{12-17}). Following digestion, samples were extracted with phenol/chloroform and ethanol precipitated.

Since the endogenous Wee1 mRNA is large (approximately 2000 nucleotides) the addition of an extended poly(A) tail could not be resolved by standard Northern blotting techniques. To test for the polyadenylation status of the endogenous Wee1 mRNA, the 3′ UTR was cleaved from the coding region by incubating 20 μg of total RNA with 0.3 μg of an antisense oligonucleotide against the 3′ end of the Wee1 coding region (GCGTC TAGAT TAATA CCCTC CGCAG GTGAA GCT) and RNase H (described above). The released 3′ UTR was separated from the remainder of the Wee1 mRNA (5′ UTR and coding region) on formaldehyde/agarose gels and analyzed by Northern blotting with a Wee 3′-UTR-specific probe.

Polyadenylation Assay Using High-Resolution Polyacrylamide Gels

Radiolabeled Wee1 UTR EMSA RNAs were synthesized with a 5′ diGTP cap. The labeled RNA was injected into immature oocytes (1–2 ng/oocyte) and total RNA was isolated after the indicated treatments, essentially as described for the Northern blot analyses. For each sample, 10 μg of total oocyte RNA was resolved on a 5% polyacrylamide (29:1), 7 M urea gel and radiolabeled RNA was visualized by autoradiography.

Electrophoretic Mobility Gel Shift Assay

Protein for gel shift assays was prepared by coupled transcription/translation in a rabbit reticulocyte lysate system using SP6 RNA polymerase (TNT Coupled System, Promega) according to the manufacturer's protocol. To generate radiolabeled RNA gel shift probes, linearized plasmids were transcribed with the SP6 RNA polymerase and 50 μM UTP, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, and 50 μCi of [α-^{32}P]UTP (400 Ci/mmol, Amersham). A total of 80 fmol of radiolabeled RNA was incubated for 20 min at room temperature with 1 μl *in vitro* transcribed and translated GST, GST–CPEB, GST–$CPEB_{1-269}$, GST–Braf, or unprogrammed rabbit reticulocyte lysate in a 20-μl final reaction volume containing 10 mM Hepes–KOH (pH 7.7), 100 mM KCl, 1 mM $MgCl_2$, 10 mM DTT, 40 μM $ZnCl_2$, 1 μg tRNA, 2 μg BSA, 8 U RNasin (Promega), 5% glycerol. A total of 100 μg heparin was then added and incubation continued for 10 min. For competition assays excess unlabeled RNA was added to incubation as indicated. Supershift assays were performed after the heparin incubation by adding 1 μl of either anti-GST or anti-BRaf (C-19) rabbit polyclonal IgG (Santa Cruz Biotechnology) to the reaction and incubating at room temperature for 10 min. After the addition of gel loading buffer, samples were run immediately on a 4% native polyacrylamide gel (30:0.8 acrylamide:bisacrylamide) at 200 V in 0.5× TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA) until the bromophenol blue dye reached the bottom of the gel. The gel was dried and exposed to film. Supershifted complexes demonstrated significantly retarded migration but did enter the polyacrylamide gel.

RESULTS

Progesterone Stimulates the Polyadenylation of the Maternal Wee1 mRNA

Previous studies have demonstrated that Wee1 protein is absent in immature oocytes but it is present in fully mature oocytes (eggs) (Murakami and Vande Woude, 1998; Nakajo *et al.*, 2000). Wee1 protein accumulation in *Xenopus* oocytes is not attributable to changes in Wee1 protein stability (Nakajo *et al.*, 2000). Since gene transcription is repressed during oocyte maturation, and Wee1 mRNA levels remain constant throughout oogenesis and oocyte maturation (Nakajo *et al.*, 2000), we hypothesized that the observed increase in Wee1 protein may be due to induction of the translation of preexisting, masked maternal Wee1 mRNA. One translational regulatory process that may contribute to the control of Wee1 protein synthesis is progesterone-stimulated cytoplasmic polyadenylation of the Wee1 mRNA.

To test whether the Wee1 mRNA encodes determinants of progesterone-stimulated polyadenylation, immature oocytes were injected with a reporter RNA specifying the full-length 3′ UTR of Wee1 (297 nucleotides). The injected oocytes were then split into two pools and either stimulated with progesterone or left untreated. At various times after progesterone treatment, the reporter RNA was recovered and polyadenylation was analyzed by Northern blotting with a probe specific for the 3′ UTR of Wee1. Because oocytes from different frogs mature at different rates in response to progesterone, the culture times (indicated in hours) have been standardized relative to the time taken for 50% of oocytes to undergo germinal vesicle breakdown (T_{GVBD50}). We define the time taken for 50% of the oocytes to undergo GVBD as $T_{GVBD50} = 1.0$. In the experiment shown in Fig. 1A, 50% of the oocytes had undergone GVBD at 3 h. Consequently samples prepared at $T_{GVBD50} = 2$ were harvested after 6 h of culture.

Oocytes that had been treated with progesterone showed a slower migration of the Wee 3′ UTR compared to that observed in immature oocytes (Fig. 1A, left panel) indicative of Wee1 3′ UTR polyadenylation. We confirmed that this retarded migration was due to the addition of an extended poly(A) tail by incubating the RNA samples from progesterone-treated oocytes with oligo(dT) and RNase H prior to the Northern analysis. This treatment selectively degrades poly(A) tails and returns the migration of the injected UTR isolated from progesterone-treated oocytes back to that observed in immature oocytes (Fig. 1A, right panel). The proportion of the Wee1 3′ UTR pool that demonstrated this retarded migration increased with time. Polyadenylation of the Wee1 3′ UTR was first observed at $T_{GVBD50} = 0.7$ (after 2 h of progesterone stimulation) when 16% of the oocytes had undergone GVBD. The polyadenylation of all the Wee1 3′ UTR transcript population was complete by $T_{GVBD50} = 4.0$, which was 12 h after progesterone treatment in this experiment (Fig. 1A, left panel).

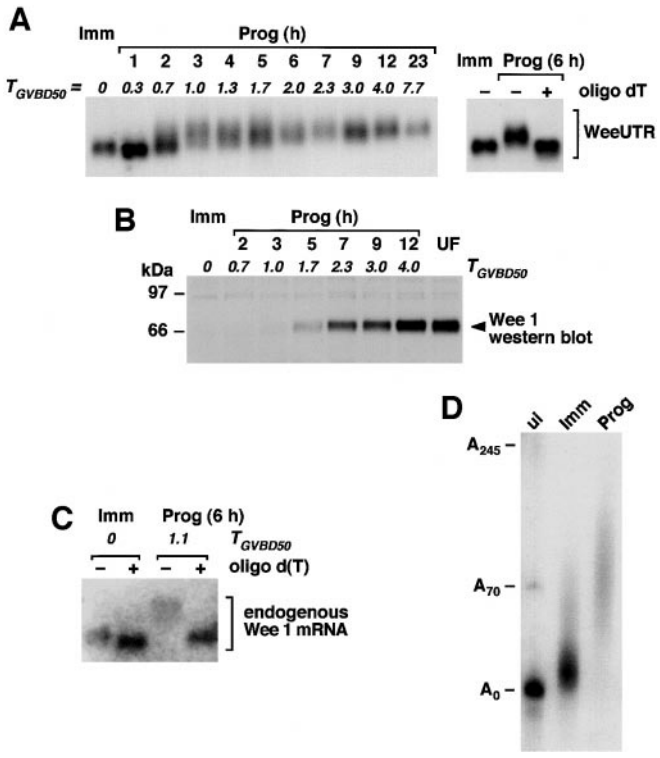

FIG. 1. Progesterone stimulates the polyadenylation of the maternal Wee1 mRNA. (A and B) The full-length Wee1 3′ UTR (297 nucleotides) was *in vitro* transcribed and injected into immature oocytes. Oocytes were then treated with (Prog) or without (Imm) progesterone and RNA was extracted from pools of 6 oocytes at the indicated times, shown as both hours (h) and standardized time T_{GVBD50} (see text). (A) RNA was extracted and resolved on a 2.2% formaldehyde/agarose gel and analyzed by Northern blot with a probe specific to the 3′ UTR of Wee1. The bracket shows the position of the Wee UTR. Retardation in the gel indicates polyadenylation. RNA from oocytes that had progesterone for 6 h were run in duplicate with one sample being treated with oligo(dT) and RNase H to remove the poly(A) tail (right panel). (B) Protein lysates were prepared and an amount of protein equivalent to one oocyte (150 μg) was resolved on a 10% Novex gel and analyzed by Western blot with anti-Wee1 antibodies as described under Materials and Methods. Expression of Wee1 is indicated by a solid arrowhead. An unfertilized egg extract (UF) was run as a positive control. (C) Immature oocytes were given progesterone for 6 h and then pools of 20 oocytes were lysed and RNA extracted. The endogenous Wee1 mRNA was cleaved by incubating with RNase H and an antisense oligo to the 3′ end of the Wee1 coding region. The released 3′ UTR was analyzed by Northern blotting with the same probe as above. The presence of a poly(A) tail was demonstrated by incubating duplicate samples with oligo(dT) and RNase H. The bracket shows the position of the endogenous, cleaved Wee1 3′ UTR. (D) High-resolution polyadenylation analysis. The last 79 nt of the Wee1 UTR were radiolabeled and injected into immature oocytes. Pools of 10 oocytes from both immature (Imm) and progesterone (Prog)-treated oocytes were lysed at T_{GVBD50} = 2 (10 h in the experiment shown). Total RNA was extracted and resolved on an polyacrylamide gel. Uninjected (ui) labeled UTR was run on the gel as a control. The length of the poly(A) tails was deduced from the migration of RNA size standards. The band in the ui lane is due to cross contamination from the adjacent marker lane.

To determine whether Wee1 protein translation coincided with the polyadenylation of the 3′ UTR, protein lysates were taken from the same set of oocytes as analyzed in Fig. 1A and the expression of Wee1 was assessed by Western blot. Figure 1B shows that there is significant Wee1 protein expression after GVBD (T_{GVBD50} = 1.7) consistent with previous reports (Murakami and Vande Woude, 1998; Nakajo *et al.*, 2000). Longer exposure of the blot revealed trace levels of Wee1 protein present at T_{GVBD50} = 1. At this time point, one-half of the oocytes had undergone GVBD and a significant proportion of the Wee1 UTR population had undergone polyadenylation. To test whether Wee1 3′ UTR polyadenylation was correlated with oocytes that had completed GVBD, progesterone-stimulated oocytes were segregated into pools based on the presence or absence of a white spot on the animal hemisphere (which is indicative of GVBD). We found that the Wee1 UTR was polyadenylated only in oocytes that had undergone GVBD (see Fig. 7B).

In order to determine whether the endogenous Wee1 mRNA was polyadenylated in maturing oocytes, immature oocytes were stimulated with progesterone for 6 h (T_{GVBD50} = 1.1) and RNA was extracted from pools of oocytes. The polyadenylation status of the endogenous Wee1 3′ UTR was analyzed by Northern blot. As can be seen in Fig. 1C, the 3′ UTR of the endogenous Wee1 mRNA is polyadenylated in progesterone-treated oocytes (compare Imm and Prog samples in the absence of oligo(dT)). When the endogenous 3′ UTR recovered from immature oocytes was incubated with oligo(dT) and RNase H, a small but significant reduction in size was also observed which probably reflects the presence of a short poly(A) tail on the Wee1 mRNA in immature oocytes.

To specifically determine the length of the Wee1 poly(A) tail in immature and progesterone-stimulated oocytes, a probe encompassing the last 79 nucleotides of the Wee1 3′ UTR was radiolabeled and injected into immature oocytes. The injected oocytes were then cultured in the presence or absence of progesterone, and total RNA was extracted after the indicated culture time and resolved on high-resolution polyacrylamide gels. The injected Wee1 3′ UTR transcripts received a short poly(A) tail in immature oocytes (Fig. 1D). This short poly(A) tail ranged from 5 to 25 residues with an average length of 10 adenylate residues. The addition of a short poly(A) tail in immature oocytes has been previously reported for other injected RNAs (de Moor and Richter, 1997; Gillian-Daniel *et al.*, 1998; Sheets *et al.*, 1994). When the injected oocytes were treated with progesterone for 10 h (T_{GVBD50} = 2) all the Wee1 3′ UTR transcripts acquired an extended poly(A) tail. The Wee1 3′ UTR transcripts received between 35 and 185 adenylate residues, with the average poly(A) length being 75 residues (Fig. 1D). Thus on average, progesterone stimulation resulted in the addition of approximately 65 adenylate residues to the Wee1 3′ UTR. Taken together, these data show that the endogenous Wee1 mRNA receives an extended poly(A) tail in progesterone-stimulated oocytes and that this polyadenylation is tempo-

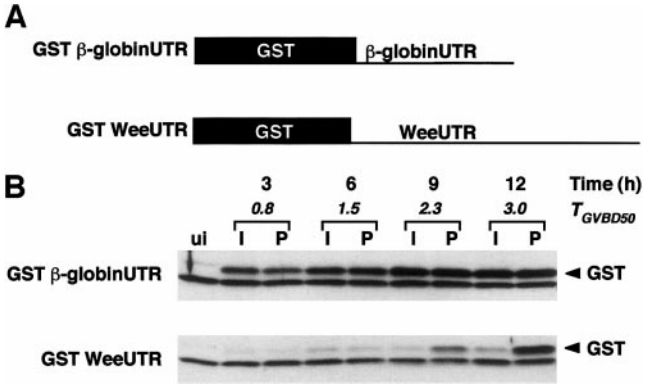

FIG. 2. The 3′ UTR of Wee1 confers translational regulation. (A) Schematic of the constructs used. The GST coding region (black box) was fused to the 154 nt 3′ UTR of β-globin or 297 nt 3′ UTR of Wee1. (B) GST reporter constructs were *in vitro* transcribed and injected into immature oocytes. Half of the oocytes were treated with progesterone (P) and the other half remained immature (I). At the indicated times pools of 10 oocytes were collected and protein lysates were prepared. 0.2 oocyte equivalent (30 μg) of protein was resolved on a 14% Novex gel and analyzed by Western blotting with GST antibodies. Expression of GST is indicated by a solid arrowhead. GST migrates slower than a cross-reacting band of unknown origin, which serves as a useful internal loading control. An uninjected oocyte sample (ui) was run as a negative control. At later time points, the level of GST protein accumulation controlled by the Wee1 3′ UTR exceeded that controlled by the β-globin UTR.

rally correlated with accumulation of the Wee1 protein after germinal vesicle breakdown.

The 3′ UTR of Wee1 Confers mRNA Translational Regulation

To determine whether the Wee1 3′ UTR directs translational control as well as the regulated polyadenylation described above, the entire Wee UTR was fused 3′ of the coding region of GST (Fig. 2A). As a control, the GST coding region was also fused to the 3′ UTR of β-globin (Fig. 2A). The β-globin 3′ UTR does not contain consensus CPE sequences, is not subject to cytoplasmic polyadenylation in maturing oocytes, and is translated in both immature and mature oocytes (Hyman and Wormington, 1988). Following *in vitro* transcription, chimeric RNA encoding either GST–Wee UTR or GST–β-globin UTR were injected into immature oocytes and then half of the oocytes were induced to mature by addition of progesterone. At the indicated times, protein lysates were taken from pools of both progesterone-stimulated oocytes and their time-matched unstimulated controls and the amount of GST protein accumulation was analyzed by Western blot (Fig. 2B). We found that GST, under the control of the β-globin 3′ UTR, accumulated continuously in a progesterone-independent fashion (Fig. 2B, top panel). When translation of GST was under the control of the Wee1 3′ UTR, the accumulation of GST protein in immature oocytes was significantly reduced compared to the levels directed by the β-globin 3′ UTR (Fig. 2B, bottom panel) indicating that the presence of the Wee1 3′ UTR causes translational repression of the GST reporter RNA in immature oocytes. The translational repression effected by the Wee1 UTR was relieved in progesterone-stimulated oocytes after 6 h of progesterone treatment (between T_{GVBD50} 1.5 and 2.3). This finding is in agreement with the accumulation of endogenous Wee1 protein (Fig. 1B) which occurred after GVBD (T_{GVBD50} = 1.7).

Three Maturation-Type CPEs Are Present within the Wee1 3′ UTR

We next wanted to identify the *cis* elements that confer translational repression and cytoplasmic polyadenylation to the Wee1 3′ UTR. Examination of the sequence of the Wee1 3′ UTR identified three candidate maturation-type CPE sequences in close proximity to the canonical AAUAAA nuclear polyadenylation hexanucleotide (Fig. 3A). One sequence, UUUUUAU, is located 25 nucleotides 5′ of the hexanucleotide (Fig. 3A, CPE 1). A second U-rich sequence, UUUUAAAU, overlaps the polyadenylation hexanucleotide (CPE 2) and the third sequence, UUUUAAU is 3′ of the polyadenylation hexanucleotide (CPE 3). In addition, a dodecauridine element was also present 30 nucleotides upstream of the polyadenylation hexanucleotide (Fig. 3A). Dodecauridine has been implicated in the control of cytoplasmic polyadenylation in embryos (Simon and Richter, 1994; Simon *et al.*, 1992, 1996) and is referred to as an embryonic-type CPE (eCPE).

Cytoplasmic polyadenylation of maternal mRNAs during oocyte maturation appears to require the CPE-binding protein, CPEB (Hake and Richter, 1994; Stebbins-Boaz *et al.*, 1996). CPEB is a sequence-specific RNA-binding protein that specifically interacts with maturation-type CPE sequences (Hake *et al.*, 1998; Hake and Richter, 1994; Stebbins-Boaz *et al.*, 1996). To determine whether the 3′ UTR of Wee1 interacts with CPEB, radiolabeled RNA specifying the last 79 nucleotides of the Wee1 3′ UTR was incubated with rabbit reticulocyte lysates expressing GST-tagged *Xenopus* CPEB. Complex formation was analyzed by EMSA. Two distinct complexes (complex A and complex B) were formed in the presence of GST–CPEB (Fig. 3B). Incubation of the probe with unprogrammed rabbit reticulocyte lysate or lysate expressing the GST moiety alone resulted in background bands, which were seen in all samples (Fig. 3B, background bands). To determine if the binding of GST–CPEB to the Wee1 3′ UTR was CPE-sequence specific we utilized RNA comprising the last 48 nucleotides of the *Xenopus* Mos 3′ UTR as a competitor for CPEB binding. The *Xenopus* Mos 3′ UTR contains a consensus CPE sequence and has been previously shown to interact with CPEB (Stebbins-Boaz *et al.*, 1996). As can be seen in Fig. 3B (left panel) inclusion of the Mos 3′ UTR RNA dramatically reduced CPEB complex formation with the Wee1 UTR. By contrast, a *Xenopus* Mos 3′ UTR which encodes a mutant

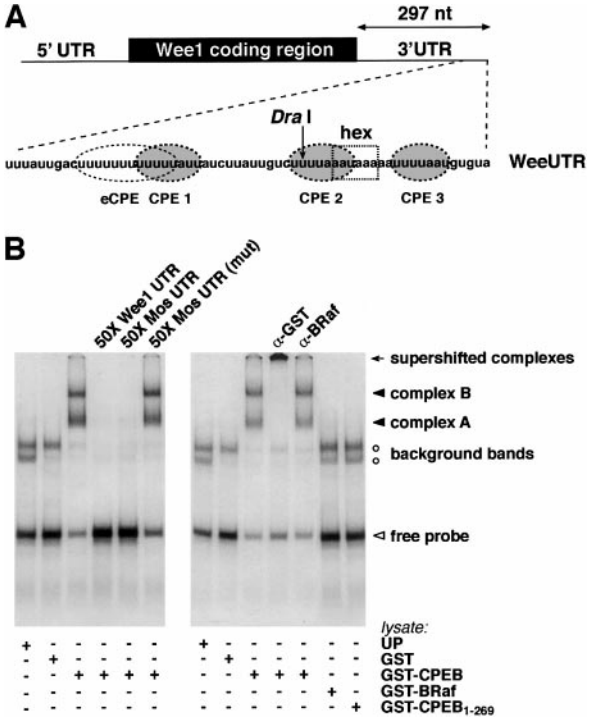

FIG. 3. The Wee1 3′ UTR specifically binds CPEB. (A) Schematic diagram showing the last 60 nucleotides of the Wee1 3′ UTR. Three maturation-type CPEs (1, 2, and 3) are marked by gray ellipses. The embryonic-type CPE (eCPE) is marked. The polyadenylation hexanucleotide (hex) is marked by a box. The natural *Dra*I restriction site is marked. (B) GST, GST–CPEB, GST–BRaf, or GST–$CPEB_{1\text{-}269}$ (does not contain the zinc finger or RNA recognition motifs) were *in vitro* transcribed/translated in rabbit reticulocyte lysates. These lysates or unprogrammed lysates (UP), as indicated, were mixed with radiolabeled RNA specifying the last 79 nucleotides of the Wee1 3′ UTR. Binding was analyzed by an electrophoretic mobility gel shift assay. Specific CPEB binding was seen as two complexes indicated by solid arrowheads and called complex A and complex B. Background bands are shown by open circles. Unbound, free probe is shown by an open arrowhead. (Left panel) Specificity of binding to the CPE was assessed by the addition of a 50-fold excess of unlabeled Wee1 3′ UTR (50× Wee1 UTR), Mos UTR (50× Mos UTR), or Mos UTR where the CPE had been mutated (50× Mos UTR (mut)). (Right panel) Specificity of CPEB binding was assessed by supershift analysis by adding GST or BRaf antibodies to the binding reaction. Supershifted complexes are shown by an arrow.

CPE (mut) did not compete for the GST–CPEB-specific complexes (Fig. 3B, left panel). Moreover, the GST–CPEB-specific complexes were competed by excess, unlabeled Wee1 UTR probe (Fig. 3B, left panel) suggesting that CPEB interacts with CPE sequences present in the Wee1 3′ UTR.

To confirm that the observed Wee1 UTR–protein complex indeed contained the GST–CPEB protein, an antibody against the GST epitope tag was used in a supershift experiment. Antiserum specific for the GST epitope tag significantly retarded the migration of complex A and complex B (Fig. 3B, right panel, "supershifted complexes") suggesting that these complexes contained GST–CPEB protein. Antiserum to an unrelated protein (BRaf), by contrast, did not retard the migration of complex A and complex B (Fig. 3B, right panel). As further evidence for the specificity of the Wee1 UTR–CPEB complex formation, we found that neither a mutant CPEB protein which lacks both RNA recognition domains and the C-terminal zinc finger (GST–$CPEB_{1\text{-}269}$) nor GST–BRaf protein was able to form a complex with the Wee1 UTR (Fig. 3B, right panel). These findings demonstrate that the formation of complexes A and B require the known RNA-binding domains and zinc finger of CPEB (Hake *et al.*, 1998) and is not attributable to the GST moiety of GST–CPEB.

A previous study has established that CPEB–CPE interactions occur in a one-to-one ratio and that monomeric CPEB binding to multiple CPEs in the same 3′ UTR does not involve any cooperative interactions (Hake *et al.*, 1998). The ability of GST–CPEB to form two complexes with the Wee1 UTR probe in the EMSA experiments (Fig. 3) suggests that several CPE sequences within the same Wee1 UTR interact independently with CPEB. To determine if all three of the candidate CPE sequences in the Wee1 UTR were *bona fide* CPEB interaction sites, a series of Wee1 3′ UTR mutants was generated to disrupt the candidate CPE sequences either individually or in combination (Table 1) and CPEB-specific complex formation was analyzed. The diguanidine mutations we introduced into the Wee1 CPE sequences are similar to those used for the mutational disruption of the CPE in the *Xenopus* G10 mRNA which completely abolished cytoplasmic polyadenylation of the G10 3′ UTR (McGrew and Richter, 1990). Similar diguanidine mutations in the CPE sequences of the cyclin B1 3′ UTR abolished CPEB binding and translational stimulation in progesterone-treated oocytes (de Moor and Richter, 1999; Hake *et al.*, 1998).

Figure 4 demonstrates that CPEB does indeed bind to all three maturation-type CPEs in the Wee1 3′ UTR. When a *Not*I restriction site disrupted eCPE and CPE 1, leaving only CPE 2 and CPE 3 intact (Fig. 4, 2&3) the formation of complex B was abolished. However, complex A was still formed indicating that CPEB interacts with either CPE 2 and/or CPE 3. When an EMSA probe was used which retains only CPE 2 (Fig. 4, 2 only), complex A was still observed. When an EMSA probe was used that only had CPE 3 intact (Fig. 4, 3 only) complex A was again observed. Since the introduction of a *Not*I site to mutate the dodecauridine eCPE also disrupted CPE 1 in the EMSA probes described above, we wanted to determine if CPE 1 was also an interaction site for GST–CPEB. To test this directly, a wild-type EMSA construct or a construct with a selective mutation in CPE 1 were linearized at a natural *Dra*I restriction site prior to *in vitro* RNA transcription (Fig. 4, designated e&1 and 'e' only, respectively). This strategy removed any contributions of CPE 2 or CPE 3 to specific complex formation. We did not detect any CPEB binding to the eCPE (Fig. 4, right panel, 'e' only), while complex A was

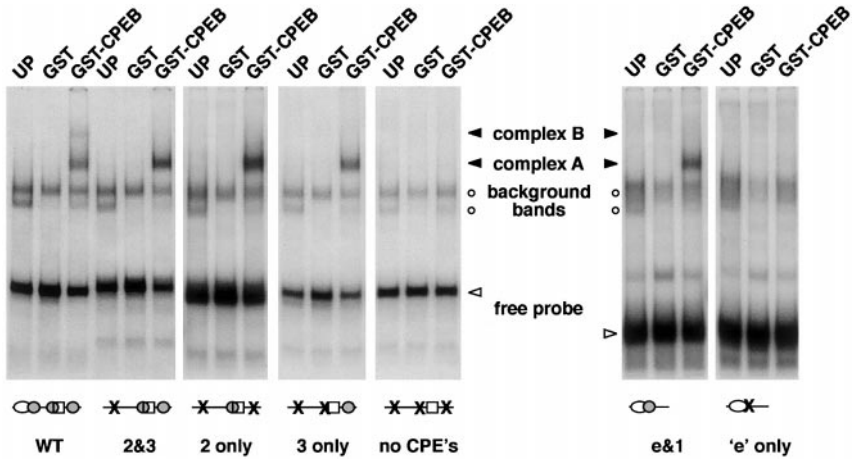

FIG. 4. CPEB binds to all three maturation-type CPE sequences in the Wee1 3′ UTR. GST or GST–CPEB were *in vitro* transcribed/translated in rabbit reticulocyte lysates. These lysates or unprogrammed lysates (UP), as indicated, were mixed with radiolabeled RNA specifying the last 79 nucleotides of wild-type or mutated versions of the Wee1 3′ UTR (see Table 1). Binding was analyzed by an electrophoretic mobility gel shift assay. Specific CPEB binding is indicated by solid arrowheads. Background bands are shown by open circles. Unbound, free probe is shown by an open arrowhead. Below each gel is a schematic of the mutations in each probe. CPEs are shown as gray circles, the eCPE is represented by an ellipse, and the polyadenylation hexanucleotide is shown as a box. Mutated elements are denoted by an "X." In mutants 2&3, 2 only, and 3 only, the 'e' and '1′ sites have been replaced by a *Not*I restriction site. In mutants e&1 and 'e' only the probe is truncated at the *Dra*I restriction site and thus migrates farther on the gel.

observed with the EMSA probe encoding both eCPE and CPE 1 (Fig. 4, right panel, e&1), indicating that CPEB can interact with CPE 1. We conclude from this observation that complex A results from the interaction of CPEB with a single CPE sequence. An EMSA probe construct that had mutations in all three CPEs (Fig. 4, no CPEs) did not form complex A indicating that no sequences in the last 79 nucleotides of the Wee1 3′ UTR, other than the identified CPEs, bind CPEB under the conditions utilized in this assay. Since the specific complex B was only observed in the wild-type EMSA probe (WT) and not the 2&3 EMSA probe (Fig. 4, left panel), we interpret this result as indicating that complex B is formed by one CPEB protein binding to CPE 1 and an additional CPEB protein binding to either CPE 2 and/or CPE 3. Consistent with this interpretation, formation of complex B was not affected by mutation of the polyadenylation hexanucleotide (which also disrupts CPE 2, see Table 1) (data not shown).

The CPE Sequences within the Wee1 3′ UTR Direct the Addition of a Long Poly(A) Tail

We next determined whether the maturation-type CPEs, which bind CPEB in the RNA EMSA experiments, actually functioned to direct cytoplasmic polyadenylation during oocyte maturation. RNA specifying the wild-type Wee1 UTR or mutant Wee1 UTRs (Fig. 5A) were injected into immature oocytes. The injected oocytes were then split into two pools, and one pool was stimulated by addition of progesterone. After 10 h, when the majority of oocytes had completed GVBD (T_{GVBD50} = 1.7), RNA was extracted from the oocytes and analyzed for addition of a poly(A) tail by Northern blot. The wild-type UTR was polyadenylated in a progesterone-dependent manner, as determined by a retarded migration in the gel, which was lost upon oligo(dT) and RNase H treatment (Fig. 5B, WT). Similarly, the mutant WeeUTR(1&2&3), which lacks the eCPE but retains all three maturation-type CPEs, was also polyadenylated in a progesterone-dependent manner. In the absence of the eCPE the mutant WeeUTR(1&2&3) received a longer poly(A) tail than the wild-type Wee1 UTR. In contrast, polyadenylation of the WeeUTR(e), which encodes mutations that disrupt all three maturation-type CPEs but retains the embryonic-type CPE, was significantly attenuated in progesterone-stimulated oocytes. Polyadenylation of the mutant WeeUTR(ϕ), which encodes mutations in the eCPE and all three maturation-type CPEs, was also significantly attenuated. However, in neither case did mutational disruption of the CPE sequences abolish progesterone-dependent polyadenylation. In the absence of maturation-type CPEs the average length of progesterone-stimulated poly(A) extension (over the length in immature oocytes) was reduced from 70 in the case of the wild-type Wee1 UTR to 5 for WeeUTR(e) and 15 in the absence of both the eCPE and the maturation-type CPEs (no CPEs) (Fig. 5C). In contrast to the CPE mutations, mutational disruption of the polyadenylation hexanucleotide (Fox *et al.*, 1989) abolished progesterone-stimulated polyadenylation (Fig. 5C). While we have not determined the relative contributions of the individual maturation-type CPE sequences, our data dem-

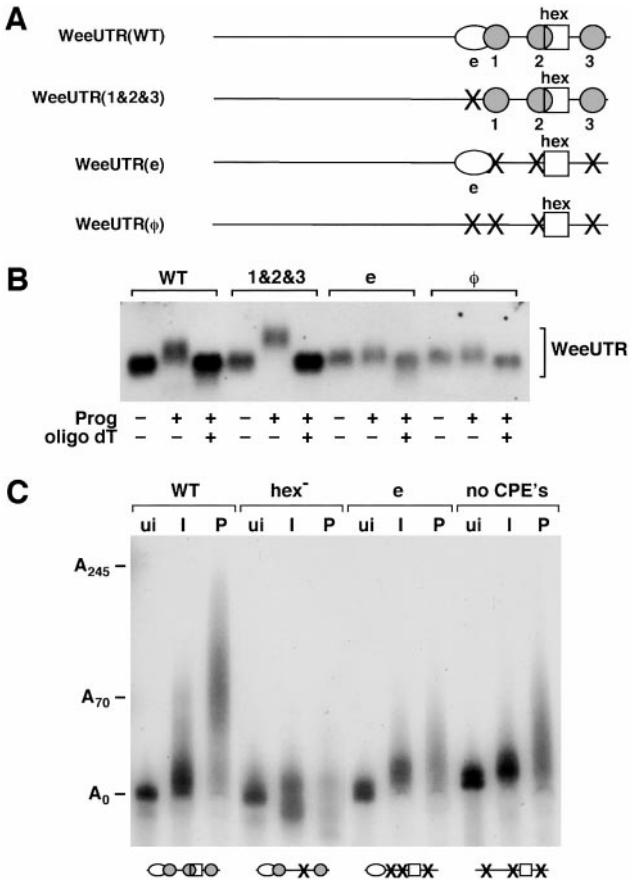

FIG. 5. Polyadenylation is regulated by maturation-type CPE sequences in the 3′ UTR of Wee1. (A) Schematic showing the CPE content of the injected UTRs. WeeUTR(1&2&3) contains only maturation-type CPEs (gray circles). WeeUTR(e) contains only the embryonic type CPE (ellipse). WeeUTR(ϕ) contains no CPEs. Mutations are marked by an "X" and are described in Table 1. (B) Wild-type (WT) Wee1 UTR and UTRs with the mutations described above were *in vitro* transcribed and injected into immature oocytes. Oocytes were given progesterone (Prog) as indicated. After 10 h (T_{GVBD50} = 1.7) pools of 10 oocytes were lysed and the RNA extracted. RNA was resolved on a 2.2% formaldehyde/agarose gel and analyzed by Northern blot with a probe specific to the 3′ UTR of Wee1. The bracket shows the position of the Wee UTR. Retardation in the gel indicates polyadenylation. Polyadenylation was confirmed by incubating duplicate samples with oligo(dT) and RNase H as indicated. (C) EMSA-sized radiolabeled RNA was injected into immature oocytes and pools of immature (I) or progesterone-stimulated (P) oocytes were lysed after 11 h (T_{GVBD50} = 2). Total RNA was extracted and resolved on a high resolution polyacrylamide gel. Uninjected (ui) RNA was run as a control. The length of the poly(A) tails were deduced from the migration of RNA size standards. A schematic diagram of the mutations in each probe is shown below the gel (see legend to Fig. 4 for symbol description).

onstrate that the CPEs in the 3′ UTR of Wee1 are necessary to mediate the addition of a long poly(A) tail to the Wee1 mRNA in progesterone-stimulated oocytes.

Distinct cis Elements within the Wee1 3′ UTR Can Confer Translational Regulation

As well as being necessary for progesterone-stimulated cytoplasmic polyadenylation, maturation-type CPEs have also been shown to mediate translational repression in immature oocytes (Barkoff *et al.*, 2000; de Moor and Richter, 1999). Therefore, we next wanted to determine whether the observed translational repression elicited by the Wee1 3′ UTR (Fig. 2) was attributable to the maturation-type CPE sequences. To address this issue, we generated chimeric RNAs where the GST coding region was fused 5′ of full-length Wee1 UTR constructs that contained a disruption of the eCPE (GST WeeUTR(1&2&3)), a disruption of all three maturation-type CPEs (GST WeeUTR(e)), or a disruption of both the eCPE and the maturation-type CPEs (GST WeeUTR(ϕ)) (see Fig. 6A). Immature oocytes were injected with RNA encoding these various chimeric constructs and then either left untreated or stimulated with progesterone. After T_{GVBD50} = 3.0 (which was after 12 h of progesterone treatment) protein lysates were made from pools of untreated or progesterone-stimulated oocytes and GST expression was analyzed by Western blot.

Consistent with the data presented in Fig. 2, the wild-type Wee UTR showed strong translational repression of GST in immature oocytes compared to the translation of the GST β-globin UTR reporter RNA (Fig. 6B, WT). Similarly, when GST was under the control of the 3′ UTR lacking the eCPE but retaining the three maturation-dependent CPE sequences, WeeUTR(1&2&3), there was strong translational repression in immature oocytes. When GST translation was regulated by Wee UTRs that contained the eCPE only or no CPE sequences at all (WeeUTR(e) and WeeUTR(ϕ), respectively), translational repression was still detected in immature oocytes when compared to expression of GST under the control of the β-globin UTR. However, in contrast to the wild-type Wee1 3′ UTR, mutant UTRs lacking the maturation-type CPEs functioned less efficiently at repressing GST translation.

Progesterone treatment relieved the inhibitory influence of the Wee1 UTR and robust progesterone-dependent translation was observed after 12 h of culture, to an extent greater than GST accumulation seen with the β-globin UTR chimera. This suggests that in addition to mediating translational repression in immature oocytes, the Wee1 3′ UTR can stimulate translation above control levels in response to progesterone. Interestingly, translational stimulation of the GST–Wee1 UTR reporter RNAs was not affected by mutational perturbation of the CPE sequences (WeeUTR(e) and WeeUTR(ϕ)). Translational stimulation exerted by a Wee1 UTR lacking eCPE was actually enhanced compared to the wild-type Wee1 3′ UTR.

To investigate the correlation between polyadenylation

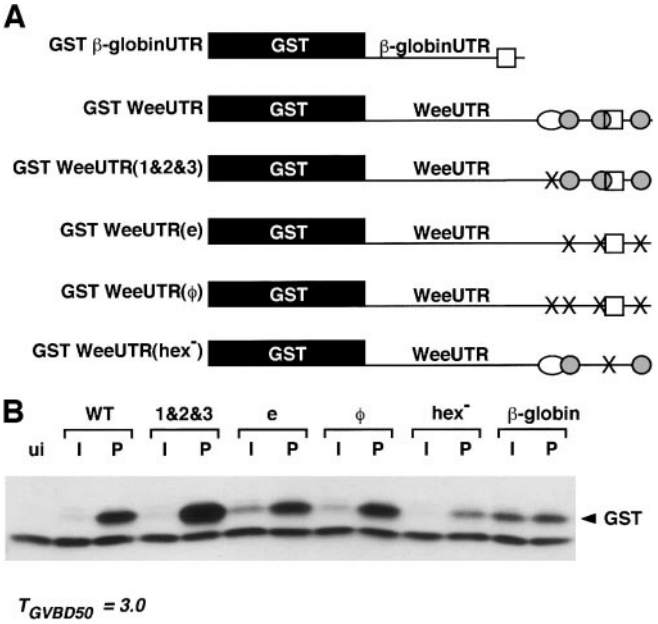

FIG. 6. Distinct *cis* elements within the Wee1 3′ UTR can confer translational regulation to a GST reporter construct. (A) Schematic diagram of the reporter constructs (see legend to Figure 4 for symbol description). (B) The GST reporter constructs were *in vitro* transcribed and the RNA was injected into immature oocytes. Protein lysates were prepared from pools of immature (I) or progesterone-treated (P) oocytes at T_{GVBD50} = 3.0 (12 h in this experiment). 0.2 oocyte equivalent (30 μg) of protein was resolved on a 14% Novex gel and analyzed by Western blotting with GST antibodies. Expression of GST is indicated by a solid arrowhead. An uninjected (ui) oocyte sample was run as a negative control.

and Wee1 translation, we examined the accumulation of GST protein from Wee1 UTRs either encoding disruptions in the CPE sequences or a disruption in the polyadenylation hexanucleotide. Disruption of the polyadenylation hexanucleotide prevented progesterone-induced translational stimulation as demonstrated by the fact that the levels of GST accumulation did not exceed the GST levels directed by the β-globin UTR (Fig. 6B). However, progesterone was able to stimulate GST translation from chimeric GST RNAs under the control of CPE-disrupted Wee1 UTRs (Fig. 6B) despite the fact that these constructs received a dramatically reduced extension of the poly(A) tail (see Fig. 5). In the particular experiment shown in Fig. 6B, the disruption of the polyadenylation hexanucleotide did not appear to affect translational repression exerted by the Wee1 3′ UTR in immature oocytes. However, we have occasionally observed reduced translational repression exerted by the mutated polyadenylation hexanucleotide Wee UTR in immature oocytes. Since the hexanucleotide mutation also disrupts CPE 2 (see Table 1), the reduction in repression may reflect the contribution of CPE 2 to this process. Taken together, our findings suggest that Wee1 3′ UTR polyadenylation is not required for translational derepression in response to progesterone but is required for translational stimulation in maturing oocytes.

CPE Sequences in the Wee1 3′ UTR Delay Progesterone-Dependent Translational Derepression until after GVBD

The accumulation of Wee1 protein in meiotically maturing oocytes is under strict temporal regulation. To investigate the role of the CPEs in the timing of translation mediated by the Wee1 3′ UTR, GST translation from chimeric RNAs with either wild-type (WeeUTR) or a mutant Wee1 UTR encoding disruptions in all CPEs (WeeUTR(ϕ)) was analyzed at various times during progesterone-stimulated maturation. Pools of oocytes were prepared following segregation into those that had undergone GVBD and those that had not. A low level of GST protein accumulation from the wild-type UTR chimera was only detected in oocytes that had completed GVBD, demonstrating that the translational derepression of the wild-type Wee1 3′ UTR occurs after completion of GVBD (Fig. 7A, top panel, compare GVBD− and + at the 5-h time point). The main induction of translation from the wild-type Wee1 3′ UTR−GST chimeric RNA occurred later, around 10 h (T_{GVBD50} = 2) which is consistent with the induction of endogenous Wee1 protein (Fig. 1). In contrast, the CPE-disrupted Wee1 3′ UTR was translationally derepressed prior to GVBD, with low levels of progesterone-dependent GST protein accumulation detectable by 4 h of culture (Fig. 7A, bottom panel). In agreement with our previous experiments, we observe less repression of GST accumulation in immature oocytes in the absence of the CPE sequences (Fig. 7A, compare the WT and ϕ-derived immature samples). An identical pattern of GST accumulation was observed when GST accumulation was controlled by a Wee1 UTR lacking the CPE sequences but retaining the dodecauridine element (WeeUTR(e), data not shown) indicating that the CPE sequences, but not the dodecauridine element, are responsible for the repression of translation prior to GVBD. A Northern analysis of the same lysates used to analyze GST protein accumulation in Fig. 7A demonstrates that the wild-type Wee1 UTR undergoes polyadenylation after GVBD (Fig. 7B, top panel, compare the GVBD− vs GVBD+ samples at T_{GVBD50} = 1) coincident with the accumulation of GST protein. Interestingly, the CPE-disrupted Wee1 UTR undergoes premature polyadenylation between 2 and 4 h after progesterone treatment, and this polyadenylation occurs prior to GVBD (Fig. 7B, bottom panel). Unlike the wild-type Wee1 UTR, the polyadenylation of the CPE-disrupted UTR appears to be transient, with deadenylation occurring between 7 and 10 h after progesterone treatment. The premature polyadenylation of the CPE-disrupted Wee1 UTR is coincident with the premature initiation of GST accumulation (Fig. 7A). Equivalent amounts of the injected RNAs were recovered from immature and progesterone treated oocytes indicating

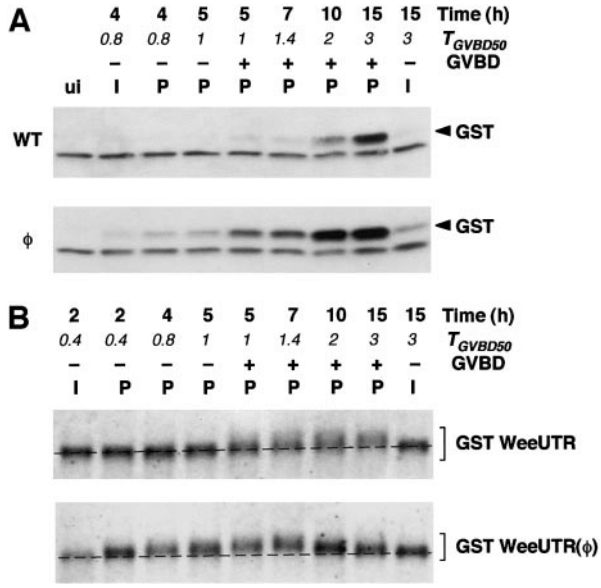

FIG. 7. CPE sequences in the Wee1 3′ UTR repress translation until after GVBD. Immature oocytes were injected with GST WeeUTR(WT) or GST WeeUTR(ϕ) (see Fig. 6A for a schematic representation of these constructs). At the indicated times, pools of either immature (I) or progesterone-treated (P) oocytes were harvested. Protein and RNA samples were sequentially prepared from each pool. Prior to harvest, oocytes were segregated based on the presence of white spots (+GVBD) or absence of white spots (–GVBD). (A) 0.2 oocyte equivalents (30 μg) of protein were resolved on a 14% Novex gel and analyzed by Western blotting with GST antibodies. Expression of GST is indicated by a solid arrowhead. An uninjected (ui) oocyte sample was run as a negative control. (B) Oocyte RNA was resolved on a 1.5% formaldehyde/agarose gel and analyzed by Northern blot with a probe specific to the GST coding region. The dashed line acts as a reference point: RNAs migrating above the line are polyadenylated.

that changes in GST accumulation (Fig. 7A) are dependent on the regulatory properties of the wild-type and CPE mutant Wee1 3′ UTRs. These results demonstrate that the CPE sequences within the Wee1 3′ UTR act to repress translation until after GVBD.

DISCUSSION

The temporal control of Wee1 activity is essential for early *Xenopus* developmental processes. Wee1 activity is inhibitory to progesterone-stimulated oocyte maturation and yet Wee1 protein must be synthesized during later phases of maturation in anticipation of the requirement for Wee1 in regulating the first embryonic cell cycle following fertilization. In this study we provide evidence that the timing of Wee1 protein accumulation is dependent upon Wee1 mRNA translational regulation. We demonstrate that CPE sequences within the Wee1 3′ UTR contribute to translational repression in immature oocytes and enforce the strict temporal regulation of translational induction in progesterone-treated oocytes.

The 3′ UTR of the Wee1 mRNA contains three maturation-type CPEs which interact with CPEB. A role for both the CPE sequences and CPEB in the translational repression of mRNAs in immature oocytes has been recently proposed (Barkoff *et al.*, 2000; de Moor and Richter, 1999; Minshall *et al.*, 1999; Stebbins-Boaz *et al.*, 1999; Stutz *et al.*, 1998; Tay *et al.*, 2000; Walker *et al.*, 1999). In support of this proposal, we find that while the wild-type Wee1 3′ UTR exerted translational repression in immature oocytes a mutant Wee1 3′ UTR with disruptions of the maturation-type CPE sequences allowed significant translation of a GST–Wee1 UTR reporter RNA. Moreover, mutational disruption of the CPE sequences resulted in premature translation of the GST–Wee1 UTR reporter RNA in response to progesterone stimulation. These findings indicate that the CPE sequences are required for the translational repression of Wee1 mRNA in immature oocytes and for translational repression during the initial stages of progesterone-stimulated oocyte maturation.

Although our data indicate a role for the CPE sequences and CPEB in the translational repression of the Wee1 mRNA in immature oocytes, the disruption of the CPE sequences did not eliminate translational repression of the chimeric GST reporter RNA (Figs. 6 and 7). These findings suggest that the Wee1 3′ UTR contains a translational repression element distinct from the CPE sequences. One potential candidate for the CPE-independent translational control sequence is the dodecauridine element within the Wee1 3′ UTR. Previous studies have demonstrated that a polyuridine element (consisting of 12 or more uridines) can direct embryo-specific cytoplasmic polyadenylation and translation (Simon and Richter, 1994; Simon *et al.*, 1992, 1996). These dodecauridine eCPE sequences have been shown to interact with *trans* factors distinct from the oocyte-type CPE binding protein, CPEB (Simon and Richter, 1994; Simon *et al.*, 1996). In the case of the Cl2 mRNA, the dodecauridine element also comprises part of a 468 nucleotide repressor element that prevents cytoplasmic polyadenylation in maturing oocytes. In our study, mutation of the eCPE in the Wee1 UTR resulted in a longer poly(A) tail extension and enhanced translational stimulation in response to progesterone (Figs. 5 and 6) which may reflect a similar repressive function of the dodecauridine element in the Wee1 UTR. However, our data do not support an obligate role for the eCPE in the regulation of Wee1 translation during oocyte maturation since this element does not contribute to translational repression of a GST reporter RNA in immature oocytes (Fig. 6, compare (e) and (ϕ)). Another example of CPE-independent translational repression has been reported for the *Xenopus* fibroblast growth factor receptor 1 (FGFR1) mRNA (Culp and Musci, 1998; Robbie *et al.*, 1995). The region within the FGFR1 3′ UTR responsible for this translational control has been defined as a 180 nucleotide translational inhibitory element (TIE)

(Robbie *et al.,* 1995). A sequence alignment between the Wee1 3′ UTR and the FGFR1 TIE revealed no overall homology. We conclude that a novel *cis* element confers translational repression to the Wee1 3′ UTR in the absence of the three CPE sequences.

In addition to the effect upon translational repression, mutational perturbation of the CPE sequences dramatically reduced the length of poly(A) extension to the Wee1 UTR in progesterone-stimulated oocytes. However, the overall level of translational stimulation of CPE-disrupted GST-Wee1 UTR reporter RNAs was not affected (Figs. 6 and 7). These findings suggest that the mutational disruptions have uncoupled the ability of the CPE sequences to mediate addition of a long poly(A) tail from progesterone-induced translational stimulation of the Wee1 3′ UTR. The translational stimulation is dependent upon the addition of a short poly(A) tail as discussed below. This poly(A) extension and translational stimulation was not attributable to the dodecauridine eCPE since mutational perturbation of this element did not affect translational stimulation and actually increased the average length of poly(A) addition (Figs. 5 and 6). The identity of the element responsible for the addition of a short poly(A) tail and for translational stimulation in response to progesterone is not known. The translational stimulation observed with the CPE-disrupted GST–Wee1 UTR in response to progesterone might reflect an aspect of the CPE which was not affected by the diguanidine "disruptional" mutations. Alternatively, a novel element, distinct from the CPE sequences may direct translational stimulation and Wee1 UTR polyadenylation in progesterone-stimulated oocytes.

The disruption of the polyadenylation hexanucleotide sequence in the Wee1 3′ UTR prevented both progesterone-induced polyadenylation and progesterone-induced translational stimulation (Fig. 6B). Our data thus support an obligate role for polyadenylation in Wee1 3′-UTR-mediated translational stimulation. Interestingly, the level of progesterone-induced translational stimulation of GST–Wee1 UTR reporter RNAs was similar regardless of whether the average poly(A) tail length was 15, 25, or 70 adenylate residues (Figs. 5 and 6). However, when the dodecauridine eCPE was disrupted the progesterone-induced average length of poly(A) tail increased to approximately 150 adenylate residues and resulted in increased GST accumulation. Our data suggest that either a poly(A) tail or the process of polyadenylation is necessary for Wee1 UTR translational stimulation and that a very long poly(A) tail (150 residues) enhances translational stimulation.

In contrast to preventing translational stimulation, the GST–Wee1 UTR chimera encoding a mutation in the polyadenylation hexanucleotide was translationally derepressed in response to progesterone resulting in a level of GST accumulation which was comparable to that directed by the β-globin UTR control (Fig. 6). This finding suggests that the derepression and translational stimulation of the Wee1 3′ UTR are separable functions. Since the polyadenylation hexanucleotide mutation prevents progesterone-induced poly(A) extension (Fig. 5) we conclude that polyadenylation is not required for the translational derepression of the Wee1 3′ UTR in response to progesterone. The dissociation of derepression and polyadenylation has been previously described for the murine tPA 3′ UTR (Stutz *et al.,* 1998). Similar to the situation we report for the *Xenopus* Wee1 3′ UTR, the translational stimulation of murine tPA requires polyadenylation. However, the dissociation of derepression and polyadenylation in the Wee1 3′ UTR differs from the derepression of the *Xenopus* cyclin B1 3′ UTR which does require polyadenylation (Barkoff *et al.,* 2000; de Moor and Richter, 1999). Our data would argue that Wee1 3′ UTR derepression, or unmasking, is independent of polyadenylation while polyadenylation is required for translational stimulation. The close temporal correlation between progesterone-induced translational initiation and the onset of polyadenylation in both the wild-type and the CPE-disrupted GST–Wee1 UTR RNAs (Fig. 7) support a model where Wee1 UTR derepression may be required for polyadenylation.

The translation of the endogenous Wee1 mRNA occurred after GVBD, at a time coincident with the polyadenylation of the Wee1 mRNA (Fig. 1). While mutational perturbation of the CPE sequences did not prevent translational stimulation (Fig. 6), the translational induction of a CPE-disrupted GST–Wee1 UTR RNA was initiated prematurely in progesterone-treated oocytes, and protein accumulation occurred prior to GVBD (Fig. 7). Based on these experiments, we propose that the CPE sequences within the Wee1 3′ UTR are the critical temporal determinants of Wee1 mRNA translation in progesterone-stimulated oocytes and function to enforce translational repression during the early stages (pre-GVBD) of maturation.

The timing of Wee1 protein accumulation during progesterone-stimulated oocyte maturation is critical since meiotic maturation requires MPF (cyclin B/cdc2) activity (Duesbery and Vande Woude, 1998; Maller, 1998). Wee1 and MPF activities are mutually antagonistic: Wee1 is a negative regulator of cdc2 and MPF can inhibit Wee1 (McGowan and Russell, 1993; Mueller *et al.,* 1995; Parker and Piwnica-Worms, 1992). Ectopic expression of Wee1 protein prior to GVBD inhibits progesterone-stimulated MPF activation and oocyte maturation (Howard *et al.,* 1999; Murakami and Vande Woude, 1998; Nakajo *et al.,* 2000). Since MPF is robustly activated at GVBD, Wee1 mRNA translation must be delayed until after progesterone-stimulated GVBD to ensure a cellular context which prejudices the balance between these two opposing activities in favor of active MPF and the inhibition of newly synthesized Wee1 protein. Indeed, it has been observed that Wee1 protein accumulates after GVBD in a hyperphosphorylated, inactive form which does not tyrosine phosphorylate cdc2 or inhibit MPF activity (Ferrell *et al.,* 1991; Mueller *et al.,* 1995; Murakami and Vande Woude, 1998). The pool of inactive Wee1 protein is reactivated following fertilization, when MPF activity is low, and functions to regulate the duration of the first embryonic cell cycle (Murakami *et al.,* 1999; Walter *et al.,*

2000). Thus, the delay in Wee1 protein accumulation until after GVBD is a critical regulatory step during progesterone-stimulated oocyte maturation.

A potential mechanism for the coordination of MPF activity and the initiation of Wee1 protein translation could be exerted through the CPE sequences in the Wee1 mRNA 3′ UTR. Previous studies have revealed that MPF activity is necessary for the cytoplasmic polyadenylation of certain maternally derived mRNAs in *Xenopus* oocytes (Ballantyne *et al.*, 1997; de Moor and Richter, 1997; Howard *et al.*, 1999). The cytoplasmic polyadenylation of the cyclin B1 mRNA is MPF-dependent and requires CPE sequences in the cyclin B1 3′ UTR (de Moor and Richter, 1997; Howard *et al.*, 1999). A potential target for MPF-dependent regulation of cytoplasmic polyadenylation is the CPEB protein which has been shown to be phosphorylated following MPF activation (Hake and Richter, 1994). While phosphorylation has little effect on the ability of CPEB to bind to CPE sequences (Hake *et al.*, 1998), CPEB phosphorylation has been correlated with CPEB degradation (de Moor and Richter, 1997; Hake and Richter, 1994). It is possible that MPF-mediated CPEB phosphorylation is required for the removal of CPEB from CPE-containing mRNAs to allow translational stimulation in maturing oocytes. The CPE-dependent temporal control of Wee1 mRNA translational initiation in maturing oocytes may be regulated by MPF activity, thus ensuring that the Wee1 protein which accumulates in maturing oocytes is inactive. In a pattern similar to that observed in *Xenopus*, the accumulation of Wee1 protein is tightly regulated during oocyte maturation in other species (Kishimoto, 1998; Mitra and Schultz, 1996). It will be interesting to determine if the temporal control of Wee1 mRNA translation is a conserved mechanism for the regulation of meiotic cell cycle progression in these other organisms.

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